

CLAIMS

1. Method to detect and identify fungal pathogenic species in a sample,
5 comprising at least the following steps:

(i) releasing, isolating and/or concentrating the nucleic acids of the fungal
pathogens possibly present in the sample,

(ii) if necessary, amplifying the Internal Transcribed Spacer region (ITS) of said
nucleic acids with at least one fungal universal primer pair,

10 (iii) hybridizing the nucleic acids of step (i) or (ii) with at least one of the
following species specific oligonucleotide probes:

GTCTAAACTTACAACCAATT (SEQ ID NO 1)
TGTCACACCAGATTATTACT (SEQ ID NO 2)
TATCAACTTGTCACACCAGA (SEQ ID NO 3)
15 GTAGGCCTTCTATATGGG (SEQ ID NO 4)
TGCCAGAGATTAAACTCAAC (SEQ ID NO 5)
GGTTATAACTAAACCAAAC (SEQ ID NO 6)
TTTTCCCTATGAACTACTTC (SEQ ID NO 7)
AGAGCTCGTCTCTCCAGT (SEQ ID NO 8)
20 GGAATATAGCATATAGTCGA (SEQ ID NO 9)
GAGCTCGGAGAGAGACATC (SEQ ID NO 10)
TAGTGGTATAAGGCGGAGAT (SEQ ID NO 11)
CTAAGGCGGTCTCTGGC (SEQ ID NO 12)
GTTTTGTTCTGGACAAACTT (SEQ ID NO 13)
25 CTTCTAAATGTAATGAATGT (SEQ ID NO 14)
CATCTACACCTGTGAACTGT (SEQ ID NO 15)
GGACAGTAGAGAATATTGG (SEQ ID NO 16)
GGACTTGGATTTGGGTGT (SEQ ID NO 17)
GTTTACTGTACCTTAGTTGCT (SEQ ID NO 18)
30 CCGCCATTCATGGCC (SEQ ID NO 19)
CGGGGGCTCTCAGCC (SEQ ID NO 20)
CCTCTCGGGGGCGAGCC (SEQ ID NO 21)

CCGAGTGCGGCTGCCTC (SEQ ID NO 22)
 CCGAGTGCGGGCTGC (SEQ ID NO 23)
 GAGCCTGAATACCAAATCAG (SEQ ID NO 24)
 GAGCCTGAATACAAATCAG (SEQ ID NO 25)
 GTTGATTATCGTAATCAGT (SEQ ID NO 26)
 GCGACACCCAACCTTTATT (SEQ ID NO 27)
 ATGCTAGTCTGAAATTCAAAG (SEQ ID NO 28)
 GGATTGGGCTTTGCAAATATT (SEQ ID NO 29)
 TTCGCTGGGAAAGAAGG (SEQ ID NO 30)
 GCTTGCCTCGCCAAAGGTG (SEQ ID NO 31)
 TAAATTGAATTTTCAGTTTATAGAATT (SEQ ID NO 32)
 TTGTCACACCAGATTATTACTT (SEQ ID NO 33),
 GGTATCAACTTGTACACCAGA (SEQ ID NO 34),
 GGTATCAACTTGTACACCAGATT (SEQ ID NO 35),
 GGTTATAACTAAACCAAACCTTTT (SEQ ID NO 36),
 GGGAATATAGCATATAGTCGA (SEQ ID NO 37),
 GGTTTTGTTCTGGACAACTT (SEQ ID NO 38),
 CATCTACACCTGTGAAGTGT (SEQ ID NO 39),
 CCGACACCCAACCTTTATTTT (SEQ ID NO 40),
 GTTGATTATCGTAATCAGT (SEQ ID NO 41),
 GAACTCTGTCTGATCTAGT (SEQ ID NO 42),
 GTCTGAATATAAAATCAGTCA (SEQ ID NO 43),

or variants of said probes, said variants differing from the sequences cited above by the deletion and/or addition of one or two nucleotides at the 5' and/or 3' extremity of the nucleotide sequence, without affecting the species specific hybridization behaviour of the probe,

or the RNA equivalents of said probes, wherein T is replaced by U,
 or the complementary molecules of said probes, and

(iv) detecting the hybridization complexes formed in step (iii).

2. Method according to claim 1, wherein the ITS region in step (ii) is limited to the ITS-1 region, and wherein the probes in step (iii) are chosen from the following set of probes:

GTCTAAACTTACAACCAATT (SEQ ID NO 1),
TGTCACACCAGATTATTACT (SEQ ID NO 2),
TATCAACTTGTACACCAGA (SEQ ID NO 3),
GTAGGCCTTCTATATGGG (SEQ ID NO 4),
TGCCAGAGATTAACTCAAC (SEQ ID NO 5),
GGTTATAACTAAACCAAAC (SEQ ID NO 6),
TTTTCCCTATGAACTACTTC (SEQ ID NO 7),
AGAGCTCGTCTCTCCAGT (SEQ ID NO 8),
GGAATATAGCATATAGTCGA (SEQ ID NO 9),
GAGCTCGGAGAGAGACATC (SEQ ID NO 10),
GTTTTGTTCTGGACAACTT (SEQ ID NO 13),
CTTCTAAATGTAATGAATGT (SEQ ID NO 14),
CATCTACACCTGTGAACTGT (SEQ ID NO 15),
GGACAGTAGAGAATATTGG (SEQ ID NO 16),
GTTTACTGTACCTTAGTTGCT (SEQ ID NO 18),
CCGCCATTTCATGGCC (SEQ ID NO 19),
CGGGGGCTCTCAGCC (SEQ ID NO 20),
CCTCTCGGGGCGAGCC (SEQ ID NO 21),
CCGAGTGCGGCTGCCTC (SEQ ID NO 22),
CCGAGTGCGGGCTGC (SEQ ID NO 23),
GAGCCTGAATACCAAATCAG (SEQ ID NO 24),
GAGCCTGAATACAAATCAG (SEQ ID NO 25),
GTTGATTATCGTAATCAGT (SEQ ID NO 26),
GCTTGCCTCGCCAAAGGTG (SEQ ID NO 31),
TAAATTGAATTTTCAGTTTTAGAATT (SEQ ID NO 32),
TTGTCACACCAGATTATTACTT (SEQ ID NO 33),
GGTTTATCAACTTGTACACCAGA (SEQ ID NO 34),
GGTATCAACTTGTACACCAGATT (SEQ ID NO 35),
GGTTATAACTAAACCAAACCTTTT (SEQ ID NO 36),

GGGAATATAGCATATAGTCGA (SEQ ID NO 37),
GGTTTTGTTCTGGACAACTT (SEQ ID NO 38),
CATCTACACCTGTGAACTGTTT (SEQ ID NO 39),
GTTGATTATCGTAATCAGTT (SEQ ID NO 41),
5 GAACTCTGTCTGATCTAGT (SEQ ID NO 42),
GTCTGAATATAAAATCAGTCA (SEQ ID NO 43),

or variants of said probes, said variants differing from the sequences cited
above by the deletion and/or addition of one or two nucleotides at the 5' and/or 3'
extremity of the nucleotide sequence, without affecting the species specific
10 hybridization behaviour of the probe,

or the RNA equivalents of said probes, wherein T is replaced by U,
or the complementary nucleic acids of said probes.

3. Method according to claim 1, wherein said fungal universal primer pair is
chosen from the following group of primer pairs:

ITS5: GGAAGTAAAAGTCGTAACAAGG and
ITS4: TCCTCCGCTTATTGATATGC,

ITS5: GGAAGTAAAAGTCGTAACAAGG and
20 ITS2: GCTGCGTTCTTCATCGATGC,

ITS1: TCCGTAGGTGAACCTGCGG and
ITS4: TCCTCCGCTTATTGATATGC,

ITS1: TCCGTAGGTGAACCTGCGG and
25 ITS2: GCTGCGTTCTTCATCGATGC,

ITS3: GCATCGATGAAGAACGCAGC and
30 ITS4: TCCTCCGCTTATTGATATGC.

4. Method according to any of claims 1 to 3, wherein said fungal pathogen is a *Candida* species, and wherein the probes of step (iii) are chosen from among SEQ ID NO 1, 2 and 3 for *C. albicans*, SEQ ID NO 4 and 5 for *C. parapsilosis*, SEQ ID NO 6 for *C. tropicalis*, SEQ ID NO 7 and 8 for *C. kefyr*, SEQ ID NO 9 for *C. krusei*, SEQ ID NO 10 for *C. glabrata*, and SEQ ID NO 11, 12 and 13 for *C. dubliniensis*.

5. Method according to claim 4 to detect *Candida albicans* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 1, 2, 3, 33, 34 and 35, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *C. albicans* in the sample.

6. Method according to claim 4 to detect *Candida parapsilosis* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 4 and 5, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *C. parapsilosis* in the sample.

7. Method according to claim 4 to detect *Candida tropicalis* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 6 and 36, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *C. tropicalis* in the sample.

8. Method according to claim 4 to detect *Candida kefyr* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 7 and 8, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *C. kefyr* in the sample.

9. Method according to claim 4 to detect *Candida krusei* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 9 and 37, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *C. krusei* in the sample.

10. Method according to claim 4 to detect *Candida glabrata* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to a probe represented by SEQ ID NO 10, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *C. glabrata* in the sample.

11. Method according to claim 4 to detect *Candida dubliniensis* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 11, 12, 13 and 38, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *C. dubliniensis* in the sample.

12. Method according to any of claims 1 to 3, wherein said fungal pathogen is an *Aspergillus* species, and wherein the probes of step (iii) are chosen from among SEQ ID NO 18, 19 and 20 for *A. flavus*, SEQ ID NO 21 for *A. versicolor*, SEQ ID NO 22, 23, 24 and 25 for *A. nidulans*, and SEQ ID NO 26 and 27 for *A. fumigatus*.

5

13. Method according to claim 12 to detect *Aspergillus flavus* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 18, 19, 20 and 42, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *A. flavus* in the sample.

14. Method according to claim 12 to detect *Aspergillus versicolor* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 21 and 43, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *A. versicolor* in the sample.

15. Method according to claim 12 to detect *Aspergillus nidulans* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 22, 23, 24, and 25, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *A. nidulans* in the sample.

16. Method according to claim 12 to detect *Aspergillus fumigatus* in a sample, said method comprising

(i) hybridizing the nucleic acids present in the sample to at least one probe chosen from among SEQ ID NO 26, 27, 40 and 41, and

(ii) detecting the hybridization complexes formed, the presence of said hybridization complexes being indicative for the presence of *A. fumigatus* in the sample.

5

17. Method according to any of claims 1 to 3, wherein said fungal pathogen is *Cryptococcus neoformans*, and wherein the probes of step (iii) are chosen from among SEQ ID NO 14, 15, 16 and 17.

10

18. Method according to any of claims 1 to 3, wherein said fungal pathogen is *Pneumocystis carinii*, and wherein the probes of step (iii) are chosen from among SEQ ID NO 28, 29, 30, 31 and 32.

15

19. Method according to any of ^{claim 1}claims 1 to 18 wherein the probes of step (iii) are immobilized to a solid support.

20

20. Method according to claim 1 for the simultaneous detection and differentiation of at least two fungal pathogenic species in one single assay, including

(i) releasing, isolating and/or concentrating the nucleic acids of the fungal pathogens possibly present in the sample,

(ii) amplifying the Internal Transcribed Spacer region (ITS) of said nucleic acids with at least one fungal universal primer pair,

(iii) hybridizing the nucleic acids of step (i) or (ii) with at least two of the following species specific oligonucleotide probes:

GTCTAAACTTACAACCAATT (SEQ ID NO 1)

TGTCACACCAGATTATTACT (SEQ ID NO 2)

TATCAACTTGTCACACCAGA (SEQ ID NO 3)

GTAGGCCTTCTATATGGG (SEQ ID NO 4)

30

005160 29429960

TGCCAGAGATTAACTCAAC (SEQ ID NO 5)
GGTTATAACTAAACCAAAC (SEQ ID NO 6)
TTTTCCCTATGAACTACTTC (SEQ ID NO 7)
AGAGCTCGTCTCTCCAGT (SEQ ID NO 8)
5 GGAATATAGCATATAGTCGA (SEQ ID NO 9)
GAGCTCGGAGAGAGACATC (SEQ ID NO 10)
TAGTGGTATAAGGCGGAGAT (SEQ ID NO 11)
CTAAGGCGGTCTCTGGC (SEQ ID NO 12)
GTTTTGTTCTGGACAACTT (SEQ ID NO 13)
10 CTTCTAAATGTAATGAATGT (SEQ ID NO 14)
CATCTACACCTGTGAACTGT (SEQ ID NO 15)
GGACAGTAGAGAATATTGG (SEQ ID NO 16)
GGAATTGGATTTGGGTGT (SEQ ID NO 17)
GTTTACTGTACCTTAGTTGCT (SEQ ID NO 18)
15 CCGCCATTCATGGCC (SEQ ID NO 19)
CGGGGGCTCTCAGCC (SEQ ID NO 20)
CCTCTCGGGGGCGAGCC (SEQ ID NO 21)
CCGAGTGCGGCTCCCTC (SEQ ID NO 22)
CCGAGTGCGGGCTGC (SEQ ID NO 23)
20 GAGCCTGAATACCAAATCAG (SEQ ID NO 24)
GAGCCTGAATACCAAATCAG (SEQ ID NO 25)
GTTGATTATCGTAATCAGT (SEQ ID NO 26)
GCGACACCCAACCTTATT (SEQ ID NO 27)
ATGCTAGTCTGAAATTCAAAG (SEQ ID NO 28)
25 GGATTGGGCTTTGCAAATATT (SEQ ID NO 29)
TTCGCTGGGAAAGAAGG (SEQ ID NO 30)
GCTTGCCTCGCCAAAGGTG (SEQ ID NO 31)
TAAATTGAATTTAGTTTGAATT (SEQ ID NO 32)
TTGTCACACCAGATTATTACTT (SEQ ID NO 33),
30 GGTTTATCAACTTGTACACCAGA (SEQ ID NO 34),
GGTATCAACTTGTACACCAGATT (SEQ ID NO 35),
GGTTATAACTAAACCAAACCTTTT (SEQ ID NO 36),

005160 29129960

GGGAATATAGCATATAGTCGA (SEQ ID NO 37),
GGTTTTGTTCTGGACAAACTT (SEQ ID NO 38),
CATCTACACCTGTGAACTGTTT (SEQ ID NO 39),
CCGACACCCAACTTTATTTTT (SEQ ID NO 40),
GTTGATTATCGTAATCAGTT (SEQ ID NO 41),
GAACTCTGTCTGATCTAGT (SEQ ID NO 42),
GTCTGAATATAAAATCAGTCA (SEQ ID NO 43),

or variants of said probes, said variants differing from the sequences cited above by the deletion and/or addition of one or two nucleotides at the 5' and/or 3' extremity of the nucleotide sequence, without affecting the species specific hybridization behaviour of the probe,

or the RNA equivalents of said probes, wherein T is replaced by U,
or the complementary molecules of said probes,

wherein said probes have been immobilized to a solid support on specific locations,

(iv) detecting the hybridization complexes formed in step (iii),

(v) identifying the species present in the sample by the location of the hybridization signal on the solid support.

21. Isolated oligonucleotide molecule having a nucleotide sequence represented by any of SEQ ID NO 1 to 43, or a variant sequence, said variant sequence differing from the sequences cited above by the deletion and/or addition of one or two nucleotides at the 5' and/or 3' extremity of the nucleotide sequence, without affecting the species specific hybridization behaviour of the molecule containing said sequence.

22. Isolated oligonucleotide molecule according to claim 21, for use as a species specific primer or probe in the detection of one of the following fungal pathogens: *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida kefyr*, *Candida krusei*, *Candida glabrata*, *Candida dubliniensis*, *Aspergillus flavus*, *Aspergillus versicolor*, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Pneumocystis carinii*.

23. Method according to claim 1, wherein the sample is a blood sample, and wherein step (i) comprises

- incubation of the blood sample with lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 50mM NaCl), followed by centrifugation and removal of the supernatant, and
- vortexing of the resuspended cell pellet in the presence of glass beads.

add Bz

005760" 25429560